CLOSTRIDIUM BOTULINUM

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After the relation of Clostridium botulinum to food poisoning had been established, the attention of bacteriologists was directed toward a better understanding of the characteristics of the causal organism. Practically all of our information rested on data in the publications of van Ermengem. Many of the observations do not seem to hold for the strains which have been isolated in America. The object of our work was to seek information concerning the reaction to heat and the natural habitat of the organism. It is one of the many which will have to be carried out before we can understand Clostridium botulinum as well as some other pathogenic anaerobes.

I. RESISTANCE OF SPORES OF CLOSTRIDIUM BOTULINUM TO DRY HEAT

Since Clostridium botulinum forms spores which are resistant to moist heat, it was thought necessary to determine whether or not the dry heat methods of sterilization are effective in destroying the spores. Tests were made on five of the cultures isolated from various sources.

A rich spore culture in brain medium was used. The culture tubes were closed with sterile corks and put on the shaking machine for about 5 minutes. This was done in order to secure as even distribution of spores throughout the medium as possible. Sterile tubes plugged with cotton were swabbed out with these cultures. By this method approximately the same number of spores was present in each tube. These tubes were inverted into large beakers, the bottoms of which were covered by several thicknesses of sterile filter paper. The plugs taken from the tubes were put into a tin receiving can which was covered and which had been sterilized for several hours. After the moisture had drained from the tubes, the sterile plugs were replaced and the tubes were then ready to be heated. A gas oven of the Lautenschläger type which is used for sterilizing glass ware in the laboratory was used. Some difficulty was experienced at first in keeping the temperature constant since a variation in gas pressure is inevitable in a large labora-

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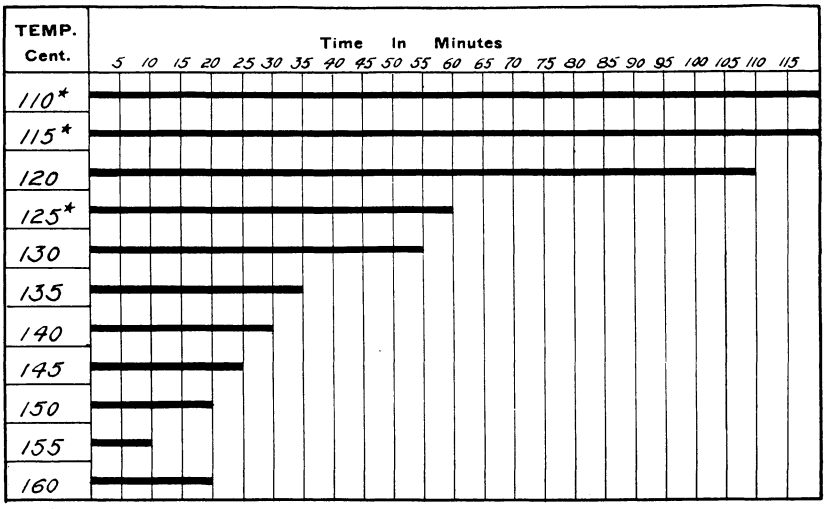

Chart 1.—Culture 7-p-1. All spores were 10 days old. The asterisks indicate that the spores survived the period of observation.

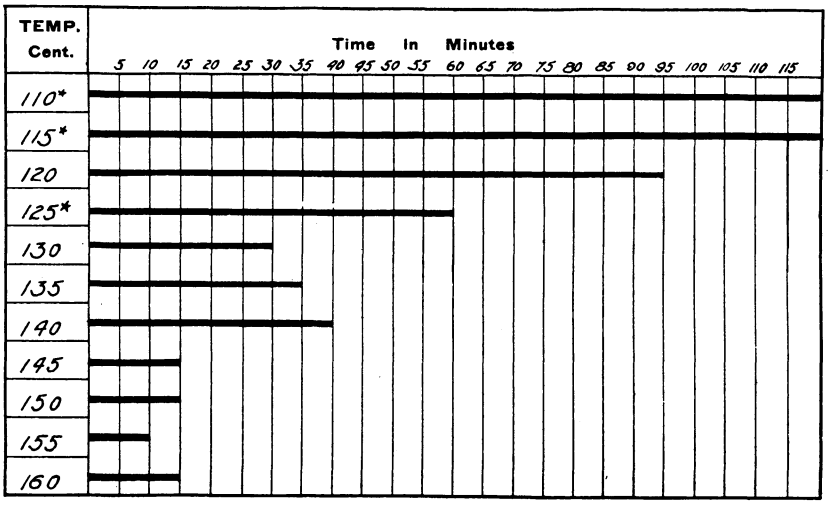

Chart 2.—Culture 2-9. All spores were 10 days old. The asterisks indicate that the spores survived the periods of observation.

tory in which many burners are in use. As the study progressed, however, it became easier to maintain a given temperature within narrow limits.

In making a test the oven was first regulated to the temperature at which the test was to be carried out. At the lower temperature the oven could not be regulated more closely than within 2 to 3 degrees of the temperature desired. Tubes were removed at various intervals. They were allowed to cool and sterile dextrose broth (9 c c to a tube) was added, after which they were sealed with parawax and incubated at 37 C. for a period sufficient to allow the development of viable spores. Viable spores were indicated by turbidity of the culture medium, formation of gas which sometimes forced the parawax upward in the tube, and by feeding tests for the presence of toxin. It was frequently noticed that growth was delayed in some of the tubes, probably due to injured spores becoming slowly viable.

The results of exposure of botulinus spores to dry heat are indicated in the charts presented herewith. It will be seen that, as would be expected, the spores survived longer at the lower temperatures. The charts for cultures 4-5, 2-9, and 7-p-1 show a progressive decrease in resistance under the conditions which obtained in the experiments. Cultures G7870, and 3p-9 show more irregular reactions, especially when compared with the other 3 cultures. For all the tests homogeneous spore suspension of the same ages were used to insure comparable data. Two exceptions were made in cultures No. 7870 and 3p-9 for the tests made at 135 C. The ages of the spores are given under the charts.

II. DISTRIBUTION IN SOIL AND FECES

The distribution of the spores of *Clostridium botulinum* is not well understood. Several investigations have been reported but more will be necessary before our information is firmly established.

Burke[1] isolated Clostridium botulinum from various sources. Cultures were made in double strength beef infusion broth with 2% glucose. Oil stratification was used to insure anaerobiosis. Other factors were also considered. At the end of the incubation period the culture was filtered and 1 c c of the filtrate was injected, subcutaneously, into a guinea-pig, 235 cultures were made from samples of various materials collected in 5 localities in central California, 50 or more miles distant from each other. Seven cultures of Clostridium botulinum were found. Clostridium botulinum was isolated from the following: bruised moldy cherries, bird pecked cherries, pole bean leaf covered with spots or droppings of insects or small animals, spiders from bush bean plants, bush beans, some of which were slightly scarred, picked over, washed and packed in

[1] Jour. Bacteriol., 1919, 4, p. 541.

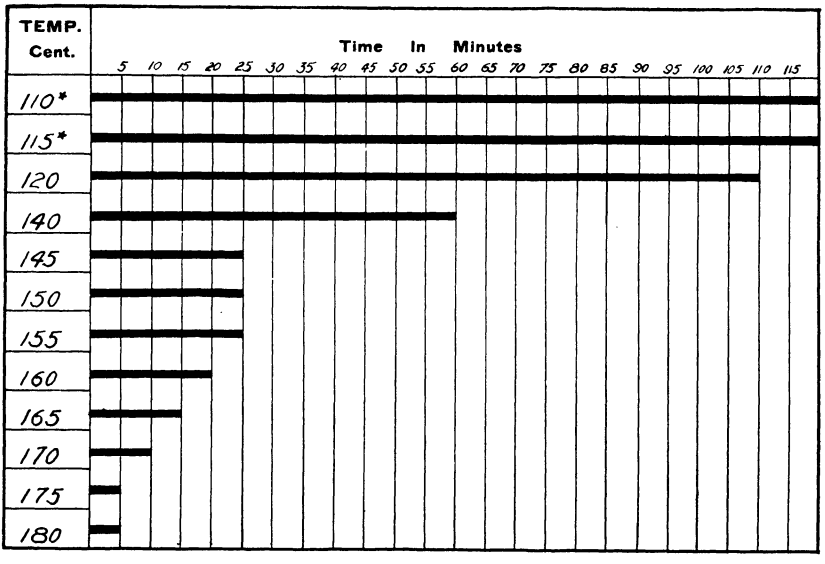

Chart 3.—Culture 4-5. All spores were between 10 and 20 days old. The asterisks indicate that the spores survived the period of heating.

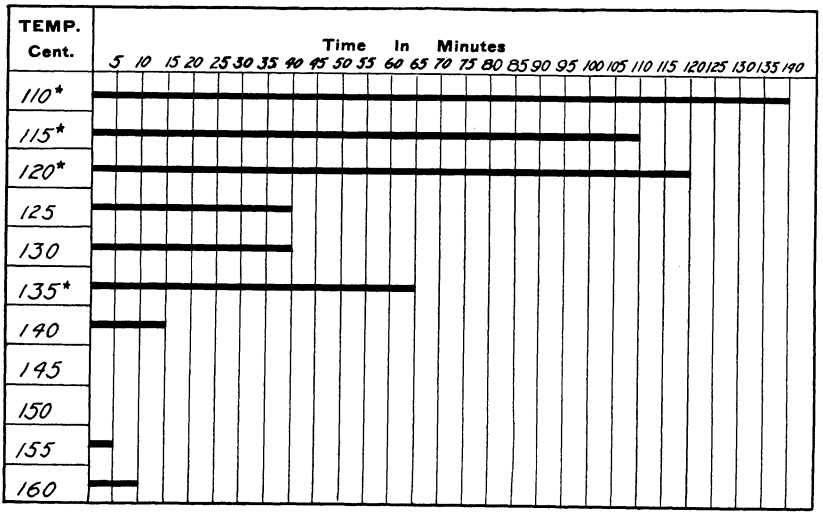

Chart 4.—Culture 3 p-9. All spores were 90 days old with the exception of those used at 135 C. which were 10 days old. The increased resistance of young spores compared with old spores is brought out. The asterisks indicate that the organisms survived the period of heating.

clean jars for canning; manure from a large hog which had recovered from botulism 3 months before the sample was taken and from discolored moldy hay from an outdoor stack.

Meyer and Geiger[2] believe that the spores may be widely distributed in nature in certain localities. Thus, it may also be found on fruits, vegetables, etc., raised in these localities. They suggest the possibility of certain animals being spore carriers. This opinion seems to be borne out by the present study.

Thirty-three samples of soils were collected from numerous places around Urbana: gardens, pastures, hog lots, corn fields, oat fields, etc. Twenty-two samples, collected from various parts of the state, were secured from Prof. R. Graham of the Department of Animal Pathology.

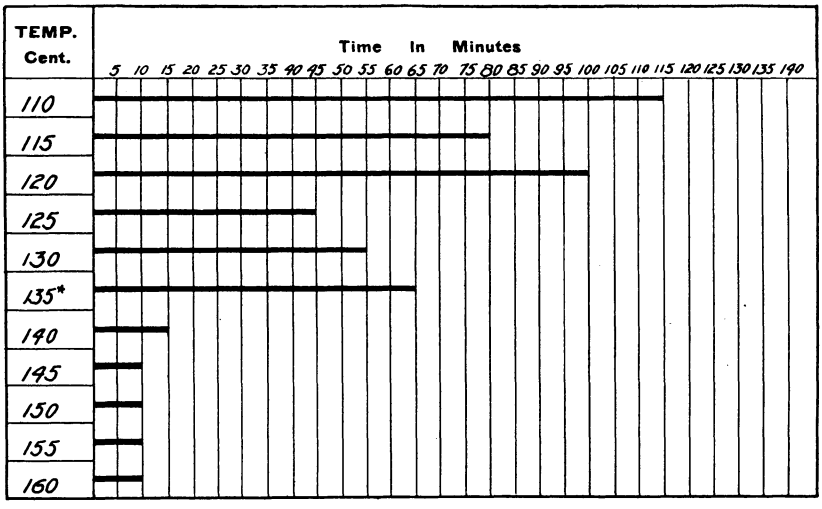

Chart 5.—Culture G 7870. All spores used were 90 days old with the exception of those used at 135 C. These spores were 10 days old. The increased resistance of these young spores is clearly brought out. The asterisk indicates that the spores survived the period of heating employed.

The technic first used was improved and modified slightly as the studies progressed. The samples of soil were put into sterile tubes and diluted with an equal volume of distilled water, or physiologic salt solution. They were then shaken on a shaking machine for about 20 minutes. This gave a homogeneous suspension. They were allowed to settle and the supernatant liquid was transferred to sterile tubes. The tubes were stoppered and heated in a water bath for 30 minutes at 80 C. Open tubes were used in the early part of the experiment.

[2] Pub. Health Repts., 1921, 36, p. 4.

TABLE 1

DISTRIBUTION OF CLOSTRIDIUM BOTULINUM IN SOIL AND FECES

Sample	Source	Toxin	Botulinus as Confirmed by Anti-toxin Type
4	Soil from old hog lot not in use at present time	+	B
5	Soil from dry hog lot	+	B
6	Soil from vegetable garden	+	B
7	Soil from city garden	+	B
8	Soil from garden	+	B
9	Soil from land that has not been manured within past 5 years	—	—
10	Soil from land which has received manure	—	—
11	Soil from land which has received manure	—	—
12	Soil from land that has not been manured within the past 5 years	—	—
16	Soil from experimental plot	—	—
17	Soil from vegetable garden	—	—
18	Garden soil	+	+
19	Garden soil	—	—
20	Soil from city garden	—	—
21	Soil from garden (new ground)	—	—
22, 23, 24	Garden soil	—	—
25	Soil from corn field	—	—
26	Soil from corn patch; manured land	—	—
27	Soil from land next to railroad; sown to oats	—	—
28	Soil from pasture	—	—
29	Soil from corn breeding plots; land formerly in cattle feeding lots	—	—
30	Soil from oat field	—	—
31	Soil from corn field	—	—
32	Soil from oat field	+	+
33	Soil from corn field	—	—
34	Soil from pasture	—	—
35	Soil from pea field, Northern Illinois	—	—
36	Soil from pea field, Northern Illinois	—	—
37	Soil from pea field, Northern Illinois	—	—
38	Soil from pea field, Northern Illinois	—	—
39	Soil from pea field, Northern Illinois	+	+
40	Garden soil, land manured, La Junta, Colo.	—	—
41	Garden soil, Everett, Wash.	—	—
42	Garden soil, Warsaw, N. Y.	—	—
43	Garden soil, Germania, Pa.	—	—
44	Garden soil, Portland, Ore.	—	—
45	Garden soil, Elgin, Ill.	—	—
46	Garden soil, Portland, Me.	—	—
47	Garden soil, New London, Conn.	—	—
48	Garden soil, Springfield, Mass.	—	—
49	Garden soil, Detroit, Mich.	—	—
50	Garden soil, Richmond, Va.	—	—
51	Garden soil, Winnebago, Ill.	—	—
52	Garden soil, Salem, Ore.	—	—
53	Garden soil, Halstead, Kan.	—	—
54	Garden soil, Vermont	—	—
55	Garden soil, Chepachet, R. I.	—	—
56	Garden soil, Oakland, Calif.	+	B
57	Garden soil, New Orleans, La.	—	—
G 570, 687, 1133, 1313, 1433, 1505, 2435, 2483, 4073, 4156, 4445, 5324, 6713, 7399, 7414, 7480, 7585, 7765, 8305	From Dr. Graham, samples from all over state	—	—
7870	From Dr. Graham	+	B
G 7855	From Dr. Graham	+	+

Various portions of the liquid were inoculated into brain medium prepared after the method of Dickson and Burke.[3] Varying numbers of drops of the liquid were also plated out in dextrose agar, and anaerobic conditions were obtained according to Dick's method. These anaerobic plates were then incubated for 48 hours or longer at 37 C. Sometimes this was combined with incubation at room temperature. From these plates inoculations were made from different suspicious colonies into 36 tubes of sheep brain medium. This sheep brain medium was found to be satisfactory since it seemed to contain the types of food materials most easily assimilated by Clostridium botulinum.

The tubes of brain medium were sealed with parawax and incubated at 37 C. for about 10 days. They were then examined and where gas formation was found accompanied by blackening of the medium with

TABLE 2

CLOSTRIDIUM BOTULINUM IN FECES AND SEWAGE

Sample	Source	Toxin	Botulinus as Confirmed by Anti-toxin Type
1	Feces from hog in apparently good health.....	+	B
2	Feces from hog in apparently good health.....	+	B
3	Feces from hog in apparently good health.....	+	B
13, 14, 15	Feces from test cows.....	—	—
58	Champaign, Ill., sewage.....	+	B

a characteristic putrid odor, they were regarded as suspicious and were held for animal inoculation. One c c of this brain medium was fed to guinea-pigs from a pipet. With strong toxins the animals showed symptoms in 5 or 6 hours and died in 10 hours. With the weaker toxins, however, death was delayed for several days. We noticed a great difference in the strength of toxins formed by these strains isolated from soil.

If toxin was found to be present as evidenced by the death of a guinea-pig with the usual symptoms of botulism in these animals, the type of the culture was determined by the use of types A and B antitoxin. Guinea-pigs dying with the usual symptoms of botulism were examined and cultures made from various organs. No difficulty was experienced in finding the organism in the brain tissues of animals showing latent death in many of these necropsies. Orr[4] has recently reported the isolation of the organism "from the organs of animals

[3] Jour. Amer. Med. Assn., 1918, 71, p. 518.
[4] Jour. Infect. Dis., 1922, 30, p. 118.

which have died following the administration of toxin-free spores either subcutaneously or by the mouth." When the organism was isolated from the brain of guinea-pigs dying from oral administration of sheep-brain medium culture (spores and toxin) it was again fed to guinea-pigs for the purpose of fulfilling Koch's postulates. No complete cultural studies were carried out. Strains of cultures revealed the usual clostridium rods.

The results of this investigation show that of the 73 samples of soil examined 11 showed the presence of an organism producing the usual symptoms of botulism in guinea-pigs. Seven of these cultures produced sufficiently strong toxins to cause the death of guinea-pigs in from 12 to 15 hours. All of these were found to be type B organisms. Four others, 18, 32, 39, and G7855 contained organisms producing a weak toxin which caused a delayed death in guinea-pigs. The symptoms, however, were those of botulism. These strains were not typed.

EXPERIMENTAL STUDY OF OCCURRENCE OF CLOSTRIDIUM BOTULINUM IN FECES AND SEWAGE

Six samples of animal feces and one sample of sewage were tested for the presence of Clostridium botulinum. Three specimens were from hogs and 3 from dairy cattle. All samples were fresh when taken. The samples were treated in exactly the same way as the soil samples. From table 3 it will be seen that the 3 samples from hogs were positive. Such data have an interesting bearing on the statement of van Ermengem that the organism might be a normal inhabitant of the intestinal tract of the hog. The 3 specimens from cows gave negative results. A series of specimens of human stools is in progress of examination.

The one specimen of raw sewage was found to contain the organism. This strain produced a powerful toxin which caused typical symptoms in guinea-pigs. Polyvalent antitoxin protected guinea-pigs against this antitoxin, but the use of homologous antitoxin showed it to be type B.

SUMMARY AND CONCLUSIONS

The different strains of Clostridium botulinum in this investigation showed different resistance to dry heat. This was probably due to inherent characteristics and in a large measure to the age of the cultures used. At 110 C. the time of survival averaged beyond 120 minutes. At 140 C. the variation was between 60 minutes and 15 minutes, a rather wide variation. At higher temperatures of 160 C. and 180 C.

the times of survival were short, between 5 and 15 minutes. The modern methods of dry heat sterilization seem, then, to be adequate for sterilizing apparatus which has been used for cultivating Clostridium botulinum. Young spores of Clostridium botulinum are more resistant to dry heat than old ones.

Clostridium botulinum like other pathogenic anaerobes is commonly present in nature. In this investigation 11 of 73 of the samples of soil contained it.

Three specimens of hog feces contained it, but it was not isolated from 3 specimens of cow feces. It was isolated from one sample of sewage.

Clostridium botulinum is probably a common saprophyte widespread in nature. The results of this investigation do not conflict with those of Meyer and Geiger who propose a regional distribution of the organism. It may be that it is more common in recently manured soils.

Further investigation must be carried out before a better understanding of the characteristics of Clostridium botulinum is attained. The occurrence of the organism in stools of healthy individuals is one phase of the subject which is now being investigated.